Immunity, Vol. 8, 31–41, January, 1998, Copyright ©1998 by Cell Press

Cooperation between Syk and Rac1 Leads to Synergistic JNK Activation in T Lymphocytes

Estela Jacinto, Guy Werlen, and Michael Karin*
Laboratory of Gene Regulation
and Signal Transduction
Department of Pharmacology
University of California, San Diego
9500 Gilman Drive
La Jolla, California 92093-0636

Summary

The MAP kinase (MAPK) JNK but not ERK is synergistically activated during costimulation of T cells. We examined how protein tyrosine kinases (PTKs) and GTPases differentially regulate JNK and ERK in T cells. While PTKs are not selective, small GTPases display distinct MAPK-activating functions. Whereas Ras activates ERK, Rac activates JNK. Rac cooperates with a Syk-generated signal to enhance JNK activation and appears to be at a nodal point for pathways emanating from CD28, calcineurin, and protein kinase C. AP-1– and NF-AT–dependent reporters are stimulated by Rac and Syk and are dependent on JNK. Unlike Syk, the PTK Lck activates JNK but does not cooperate with Rac, resulting in weak AP-1 and NF-AT activation. Therefore, signals generated by PTKs are functionally distinct and need to be integrated to induce transcriptional responses.

Introduction

T cells require costimulation for full activation (Schwartz, 1992). Although antigen presented by the major histocompatibility complex is recognized by the T cell receptor (TCR), leading to activation of cytoplasmic protein tyrosine kinases (PTK), these signals are not sufficient for full T cell activation (Schwartz, 1992). Additional signals provided by occupancy of auxiliary receptors, such as CD28, by ligands on the surface of antigen-presenting cells are necessary (Linsley and Ledbetter, 1993). Engagement of these receptors triggers pathways that are integrated to induce transcription of the gene for interleukin-2 (IL-2), a major T cell growth factor (Linsley and Ledbetter, 1993). Absence of costimulation leads to an anergic state in which T cells lose the ability to induce IL-2 upon restimulation (Schwartz, 1992).

Cytoplasmic PTKs, including members of the Src (Lck and Fyn) and Syk (ZAP70 and Syk) families, play a critical role in transmission of signals from the TCR complex (Weiss and Littman, 1994; Wange and Samelson, 1996). Other PTKs, such as the Tec family (ITK/EMT), have been suggested to transmit signals from CD28 (August et al., 1994). Upon activation, the Src PTKs Lck and Fyn phosphorylate immunoreceptor tyrosine-based activation motifs (ITAM) in the cytoplasmic domains of TCR subunits (Weiss and Littman, 1994). This results in recruitment of Src homology-2 (SH2)–containing proteins to the TCR complex. In some T cell subsets Syk kinases are constitutively associated with the TCR complex and, upon activation, recruit and activate other signaling molecules, including Src kinases (Thome et al., 1995). Although the exact order of events and the immediate downstream targets of these PTKs are not fully established, their importance in T cell signaling and development are well recognized. Mutations in genes encoding these PTKs impair lymphocyte signaling, development, and immune regulation (Arpaia et al., 1994; Lowell and Soriano, 1996; Groves et al., 1996; van Oers et al., 1996).

Events involved in the transmission of signals from CD28 or other auxiliary receptors remain nebulous. It also is not clear how PTK activation eventually leads to IL-2 gene induction. However, as in other cell types in which receptor PTKs transduce extracellular signals to stimulate gene transcription (Marshall, 1995), a similar scheme probably applies to cytoplasmic PTKs in T cells (Weiss and Littman, 1994). Stimulation of receptor PTKs results in activation of mitogen-activated protein kinase (MAPK) cascades via small guanosine triphosphate (GTP)–binding proteins (Karin and Hunter, 1995; Marais and Marshall, 1996). MAPKs play a critical role in transmitting signals generated by PTKs to the nucleus (Karin and Hunter, 1995; Su and Karin, 1996). Once activated in the cytoplasm, MAPKs translocate to the nucleus to phosphorylate and thereby stimulate specific transcription factors. In addition to the extracellular signal–regulated kinases (ERKs), the MAPK family includes the c-Jun N-terminal kinases (JNKs) and p38s, also known as stress-activated protein kinases (Su and Karin, 1996). Different MAPKs have distinct yet overlapping substrate specificities. For example, only the JNKs phosphorylate c-Jun, whereas both JNKs and p38s phosphorylate ATF2 (Su and Karin, 1996) and only p38 phosphorylates MEF2C (Han et al., 1997). All three groups of MAPKs phosphorylate Elk-1 (Cavigelli et al., 1995; Price et al., 1996).

In T lymphocytes, JNK is synergistically activated by costimulation of the TCR/CD3 and CD28 receptors or by combined treatment with phorbol myristate acetate (PMA) and Ca^{2+} ionophore (Su et al., 1994). In contrast to JNK, no synergy is observed in ERK activation. While ineffective in JNK activation, either TCR engagement or PMA alone can fully activate ERK. The synergistic JNK activation response is unique to T cells (Su et al., 1994) and B cells (Healy et al., 1997) and so far has not been not observed in any other cell types. The response of MAPKs to signals emanating from PTKs is dependent on small GTPases such as Ras and Rac (Coso et al., 1995; Minden et al., 1995). Ras is necessary and sufficient for ERK activation (Marais and Marshall, 1996). However, it is necessary but insufficient for JNK activation (Su and Karin, 1996). The latter is more dependent on activation of Rac or Cdc42, which are not involved in ERK activation (Coso et al., 1995; Minden et al., 1995). These findings were made in fibroblasts and epithelial cells, but the role of these proteins in T cell activation has heretofore not been identified.

*To whom correspondence should be addressed (karinoffice@ucsd.edu).

The importance of JNK for IL-2 induction is also suggested by the role of the AP-1 transcription factor, composed of Jun and Fos subunits. In addition to binding AP-1 recognition sites, AP-1 assists the Ca^{2+}-responsive, cyclosporin A (CsA)–sensitive nuclear factors of activated T cells (NF-AT_c) to bind stably the composite NF-AT recognition element in the IL-2 promoter (Rao, 1994). NF-AT_c activity is regulated mostly posttranslationally; in nonstimulated cells, the NF-AT_c proteins reside in the cytoplasm but in response to stimulation by PMA and Ca^{2+} ionophore, they translocate to the nucleus. This nuclear translocation is Ca^{2+} and calcineurin dependent and sensitive to CsA (Rao, 1994). AP-1 activity is regulated both transcriptionally and posttranscriptionally, but once synthesized, AP-1 subunits are constitutively nuclear (Karin et al., 1997). In nonstimulated T cells, the basal levels of AP-1 proteins are low but T cell activation results in induction of *jun* and *fos* genes and increased synthesis of AP-1 components (Rincon and Flavell, 1994; Su et al., 1994). This process depends on activation of JNK and other MAPKs (Su et al., 1994). In addition to stimulating preexisting factors involved in c-*jun* or c-*fos* induction, the JNKs phosphorylate newly synthesized c-Jun or JunD proteins and enhance their ability to activate transcription (Karin et al., 1997). By phosphorylating Elk-1 (Price et al., 1996) and MEF2C (Han et al., 1997), the p38 MAPKs also participate in *fos* and *jun* gene induction and stimulation of AP-1 activity.

A critical role for MAPKs in T cell activation is underscored by studies of anergic T cells that demonstrated that both JNK and ERK activation are diminished following TCR restimulation (Fields et al., 1996; Li et al., 1996). This observation accounts for a defective AP-1 response (Kang et al., 1992) and impaired expression of some AP-1 proteins in anergic T cells (Mondino et al., 1996). Hence, a better understanding of the pathways that regulate MAPKs is relevant for elucidating the mechanisms of T cell activation and anergy.

To define the pathways accounting for differential regulation of MAPKs and to identify the signals that need to be integrated for potent JNK activation, we analyzed the role of cytoplasmic PTKs and small GTPases in these processes. We show that Lck is required for both JNK and ERK activation through the CD3 receptor. While Syk may not be required for JNK activation, its presence can enhance the JNK response to CD3 and CD28 costimulation. Our results indicate that whereas Lck and Syk activate both JNK and ERK in transfection assays, Rac1 can cooperate with Syk but not Lck to cause a further increase in JNK activity. In contrast, Rac1 does not activate ERK and does not potentiate its activation by either Lck or Syk. In addition, Rac1 can couple the CD3, CD28, and protein kinase C (PKC) signals to JNK activation. Consistent with the convergence of the Syk and Rac pathways in JNK activation, the same signals also synergize to induce AP-1 and NF-AT transcriptional activities.

Results

Src and Syk PTKs Activate JNK and ERK

The MAPKs JNK and ERK are differentially regulated in T cells. Whereas ERK is efficiently activated by either TCR occupancy (anti-CD3) or PMA, JNK activity is stimulated only weakly by these agonists. Potent JNK activation requires co-occupancy of CD3 and CD28 or coincubation with PMA and Ca^{2+} ionophore. Neither CD28 occupancy nor Ca^{2+} ionophore activates JNK by itself (Su et al., 1994). PTKs that couple receptor stimulation to downstream targets may lead to activation of either JNK or ERK or both. We therefore compared the abilities of Src (Lck and Fyn) and Syk (Syk and ZAP70), PTKs that are predominantly expressed in T cells, to activate JNK and ERK. Wild-type and activated forms of Lck and Fyn (Cooke and Perlmutter, 1989; Wright et al., 1994) and wild-type Syk and ZAP70 (Chan et al., 1992; Couture et al., 1994) were tagged with the hemagglutinin (HA) epitope, so their expression could be compared. Plasmids encoding each of these PTKs were cotransfected with Flag (M2)–tagged JNK1 or HA-ERK2 expression vectors (Minden et al., 1994) into the Jurkat T cell line. We found that Lck and Syk activate JNK, but Fyn and ZAP70 were considerably less potent (Figure 1A). The observed activation of JNK by Lck or Syk, however, is incomplete when compared to the effect of PMA+A23187. Similar effects on JNK activity were also obtained using untagged PTK constructs (data not shown). We also found that untagged Lck, Fyn, and Syk activate ERK to about the same degree, which was only a bit lower than the response to PMA, the most effective ERK activator in Jurkat cells (Figure 1B).

We further investigated the role of Lck and Syk in MAPK activation. We compared the response of the Jurkat variant JCaM1.6, which is defective in Lck expression (Straus and Weiss, 1992), with that of a reconstituted derivative, JCaM1/lck, which expresses functional Lck. JCaM1/lck responds normally to TCR occupancy in terms of Ca^{2+} spikes and induction of tyrosine phosphorylation (Straus and Weiss, 1992). CD3 and CD28 expression levels were comparable in both cell lines as determined by fluorescence-activated cell sorter analysis (data not shown). Immunoblotting revealed large differences in expression of the different modified forms of Lck between the two cell lines (Straus and Weiss, 1992) (Figure 2A). Costimulation with antibodies to CD3 and CD28 resulted in synergistic JNK activation in the Lck-expressing cells, while no JNK activation was observed in the Lck-defective cell line (Figure 2A). We noticed higher basal JNK activity in the Lck^- cells, and thus the fold activation was lower in these cells compared to that of the Lck^+ cells. Lck, however, is not required for activation of JNK by PMA. When PMA was combined with A23187 or anti-CD28 in the defective cell line, synergistic JNK activation was still observed but not when anti-CD3 was used as a costimulus (Figure 2A). However, CD28 ligation potentiated the response to PMA by 7-fold in Lck^+ cells and by only 3–4-fold in Lck^- cells. These results suggest that Lck is not required for the transmission of signals generated by PMA or Ca^{2+} ionophore leading to JNK activation and that it has only a minor role in the response to CD28 occupancy.

Since overexpression of Syk can activate JNK partially (Figure 1A), we tested whether this signal can be enhanced by a second signal. Our results show that activation of JNK by Syk overexpression can be further increased upon activation with anti-CD3, anti-CD28, or PMA, but not after treatment with Ca^{2+} ionophore (Figure

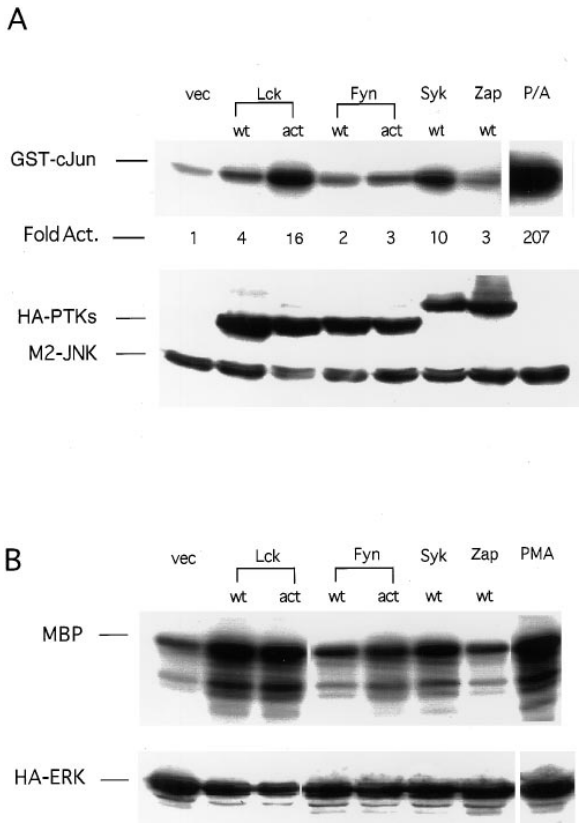

Figure 1. Lck, Fyn, and Syk Are Involved in JNK and ERK Activation

(A) To obtain equivalent expression of cytoplasmic PTKs, Jurkat Tag cells were transfected with HA-tagged wild-type Lck (1 μg), activated Lck (HSB2-A1; 3 μg), wild-type Fyn (2 μg), activated Fyn (F528; 2 μg), wild-type Syk (1.5 μg), wild-type Zap70 (0.2 μg), or SRα empty vector (3 μg), together with 0.2 μg of M2-tagged JNK. Kinase activity was measured by immune complex kinase assay using anti-M2 to immunoprecipitate JNK and GST-c-Jun(1–79) as a substrate. A sample of each lysate was analyzed for protein expression by immunoblotting with anti-HA and anti-M2. Fold activation was determined after quantitation with the phosphoimager (Bio-Rad). vec, vector; wt, wild-type, act, activated; P/A, PMA+A23187.

(B) Jurkat Tag cells were transfected with untagged PTK expression vectors (same concentrations as in [A]) together with 0.2 μg of HA-ERK expression vector. Protein expression and kinase activity, using anti-HA to immunoprecipitate HA-ERK and MBP as a substrate, were determined as described in (A).

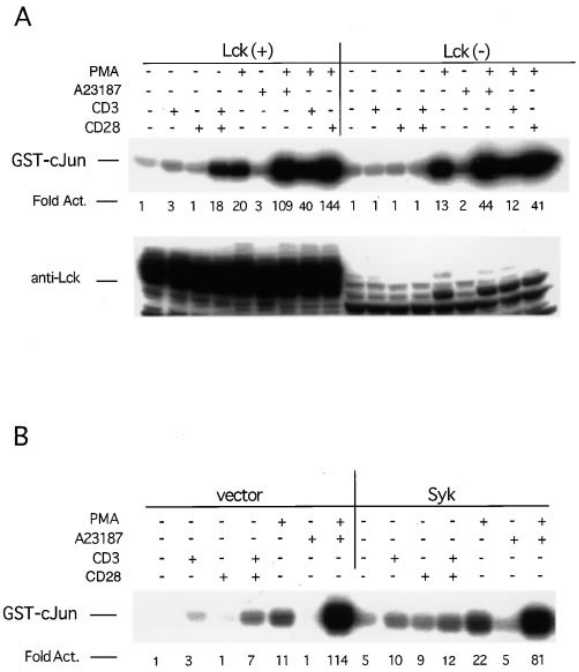

Figure 2. Role of Lck and Syk in T Cell Costimulation

(A) The Jurkat variant JCaM1.6, which expresses a defective form of Lck [Lck$^-$], and a stably transfected form that expresses wild-type Lck (JCaM/lck) [Lck$^+$] each were stimulated for 30 min as indicated. The activity of endogenous JNK immunoprecipitated with anti-JNK (333.8) was determined using GST-c-Jun(1–79) as a substrate and quantitated as in Figure 1. Expression of Lck was determined by immunoblotting. Total protein concentration was measured for each sample by Lowry assay (Bio-Rad). Fold activation of JNK was calculated after quantitating GST-c-Jun phosphorylation with a phosphoimager.

(B) Jurkat Tag cells were transfected with either 2 μg of empty vector (SRα) or a Syk expression vector together with 0.2 μg of HA-JNK1 vector. Cells in 1 ml of suspension were subjected to the indicated stimuli and collected after 30 min. After determination of the level of HA-JNK1 in each sample by immunoblotting, JNK activity was determined by an immune complex kinase assay performed on equal amounts of HA-JNK1, as described in (A).

2B). From these, most notable is the augmentation by anti-CD28. As previously described (Su et al., 1994), CD28 ligation by itself has a marginal effect on JNK activity. However, when combined with Syk overexpression, CD28 ligation activated JNK as efficiently as CD3+CD28 ligation in mock-transfected cells (Figure 2B). It should be noted, however, that none of the costimulations in Syk transfected cells resulted in full JNK activation when compared to the PMA+A23187 treatment.

Rac1 Cooperates with Syk but Not Lck to Enhance JNK Activation

Since no major difference in the pattern of MAPK activation was observed between Lck and Syk overexpression, we searched for possible modulators of their effect. The role of the small GTPases Ras and Rac in MAPK activation has been well studied in other cell types (Coso et al., 1995; Minden et al., 1995). Ras has also been shown to couple TCR signals to ERK activation in T cells (Cantrell, 1996), but the role of Rac in T cell activation is not clear. We cotransfected the dominant inhibitory Rac1(N17) mutant (Minden et al., 1995) with either Lck or Syk and an HA-JNK1 vector. We observed no significant inhibitory effect of Rac1(N17) on JNK activation by Lck and Syk (Figure 3A). These results stand in marked contrast to the ability of Rac1(N17) to inhibit JNK activation by Src in NIH3T3 or HeLa cells (Minden et al., 1995).

This led us to investigate whether in T cells Lck or Syk may act in parallel with Rac. We therefore tested whether activated Rac1 can synergize with the Lck- or Syk-generated signals. As observed in other cell types (Coso et al., 1995; Minden et al., 1995), Rac1(V12) activates JNK efficiently by itself in Jurkat cells (Figure 3B). While Lck or Syk have a modest effect on JNK activity, coexpression of Rac1(V12) potentiates JNK activation by Syk but not by Lck. In fact, Rac1(V12) can actually decrease JNK activation by Lck. We also tested whether an activated form of Ras will synergize with Lck or Syk.

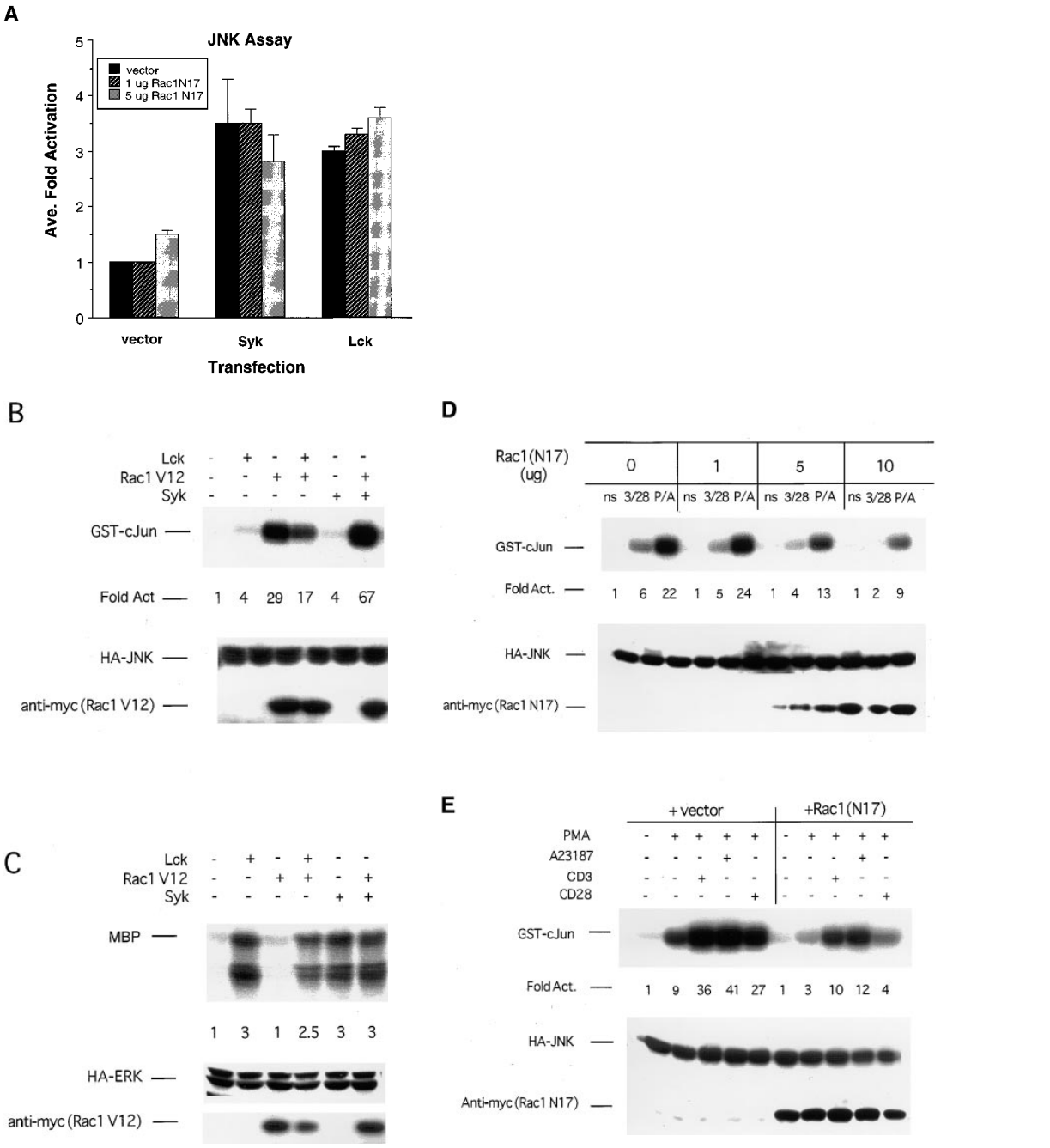

Figure 3. Rac1 Potentiates and Couples Costimulating Signals to JNK Activation

(A) Jurkat Tag cells were transfected with 2 μg of activated Lck or wild-type Syk expression vector together with 0.2 μg of HA-JNK1 and either an empty expression vector or 1–5 μg of Rac1(N17). The total amount of DNA transfected was kept constant using empty SRα expression vector. After determining and normalizing for HA-JNK1 expression, its kinase activity was measured as described for Figure 1. Expression of Myc-tagged Rac1(N17) was also examined by an immunoblot (data not shown). The average fold activation of HA-JNK1 was determined by three independent transfection experiments.

(B) Jurkat Tag cells were transfected alone or in combination with 2 μg each of expression vectors coding for activated Lck, wild-type Syk, or Rac1(V12), together with 0.2 μg of HA-JNK1 vector. After normalizing HA-JNK1 expression determined by immunoblotting, its kinase activity was measured by an immunecomplex kinase assay. Rac1(V12) expression was examined by immunoblotting.

(C) Jurkat cells were transfected as in (B) but with 0.2 μg of HA-ERK2 expression vector instead of HA-JNK. After normalizing for HA-ERK2 expression by immunoblotting, kinase activity was determined by immune complex kinase assay using MBP as a substrate.

(D) Jurkat Tag cells were transfected with increasing amounts of Rac1(N17) together with 0.2 μg of HA-JNK1. The cells were subjected to the indicated stimuli, and after 30 min lysates were prepared. JNK activity and protein expression were measured. ns, not stimulated; 3/28, anti-CD3+anti-CD28; P/A, PMA+A23187.

(E) Jurkat cells were transfected with 5 μg of either Rac1(N17) or an empty SRα expression vector and subjected to the indicated stimuli for 30 min prior to determination of JNK activity and protein expression.

Ras(L61) alone did not activate JNK significantly, nor did it synergize with either Lck or Syk even at high expression levels. Like Rac1(V12), expression of Ras(L61) attenuated the response of JNK to Lck or Syk (data not shown).

We tested the effect of Rac1(V12) on ERK activation and found that Rac1(V12) by itself did not activate ERK, nor did it potentiate ERK activation by Syk or Lck expression despite efficient expression of the protein (Figure 3C). ERK activity, however, was stimulated by Ras(L61) expression (data not shown).

We next examined whether Rac1 is involved in the costimulatory signals generated by PMA and Ca^{2+} ionophore or anti-CD3 and anti-CD28. Dominant negative Rac1(N17) was cotransfected with HA-JNK1 and the cells were costimulated with anti-CD3+anti-CD28 or PMA+Ca^{2+} ionophore. We observed diminished JNK activation in response to increasing amounts of Rac1(N17) (Figure 3D). Rac1(N17) can inhibit the response to anti-CD3+anti-CD28 almost fully. Inhibition of the response to PMA+Ca^{2+} ionophore was less complete. We further examined the effect of Rac1(N17) on JNK activation by combining PMA and other agonists. We found that Rac1(N17) can inhibit PMA-mediated JNK activation by 3-fold (Figure 3E). When PMA was combined with anti-CD3 or Ca^{2+} ionophore, the same degree of inhibition was observed. However, when the cells were costimulated with PMA+anti-CD28, as much as 7-fold inhibition of JNK activation was obtained. These results suggest that Rac1 is involved in JNK activation by PMA and is probably also involved in the costimulatory CD28 pathway.

Syk Increases AP-1 Activity

JNK phosphorylates the transactivation domain of transcription factor c-Jun, which dimerizes with other Jun and Fos family members and induces transcription from promoters containing AP-1 recognition elements (Karin et al., 1997). JNK activation also correlates with induction of c-*jun* transcription (Karin, 1995; Karin et al., 1997). We used a −79 c-*jun*-luciferase (LUC) reporter to assess the induction of c-*jun* transcription and to correlate it with activation of JNK by Lck and Syk. Cotransfection of c-*jun*-LUC with Syk results in 6-fold induction of reporter gene expression (Figure 4A). The response to Syk was not significantly increased by either PMA or Ca^{2+} ionophore (Figure 4A) and is only slightly sensitive to CsA (Figure 4B). The observed induction by Syk is not as high as the effect of PMA+Ca^{2+} ionophore. Overexpression of active Lck minimally induced c-*jun*-LUC transcription (2-fold) and was not potentiated by either PMA or ionophore (data not shown); it also was not sensitive to CsA (Figure 4B).

We also tested the effect of Rac1(V12) on c-*jun* promoter activation and examined whether it correlates with the synergistic activation of JNK when coexpressed with Syk. Rac1(V12) induced transcription of c-*jun*-LUC, and this response was potentiated by coexpression of Syk (Figure 4C). This synergistic induction as well as the lack of cooperation of Rac with Lck correlates well with the effects of these molecules on JNK activity. A reporter, −60 Col-LUC, lacking an AP-1 binding site, was not induced by expression of Syk, Lck, and Rac1 (Figure 4D).

Syk Can Induce NFAT Activity More Potently Than Lck, and This Induction Is Only Partially Sensitive to CsA

Induction of IL-2 is a key event in T cell activation. Transcription of IL-2 is controlled by several *cis*-acting elements, one of which, the NF-AT recognition site, was shown to have a lymphoid-specific role (Rao, 1994). We investigated how signals from Lck can lead to induction of NF-AT activity by using a reporter construct driven by a minimal IL-2 promoter (−72 to +47), upstream of which three NF-AT binding sites were inserted (Northrop et al., 1993). Occupancy of this NF-AT site and promoter activation require cooperative binding of $NF\text{-}AT_c$ proteins and AP-1 transcription factors (Rao, 1994). Syk expression strongly increased NF-AT-LUC expression, which was further enhanced (2-fold) by Ca^{2+} ionophore but not by PMA (Figure 5A). In fact, consistent reduction in NF-AT reporter activity was observed when Syk-transfected cells were costimulated with PMA. Costimulation with PMA and Ca^{2+} ionophore strongly induced NF-AT activity, as expected (Northrop et al., 1993), and this effect was not augmented by coexpression of Syk (Figure 5A). While the response to PMA+Ca^{2+} ionophore was almost completely inhibited by CsA, the response to Syk was only partially inhibited (250-fold inhibition of PMA+Ca^{2+} ionophore stimulation vs. 3-fold inhibition of the response to Syk) (Figure 5B).

When the activated Lck was cotransfected with the NF-AT-LUC reporter, we observed only a 10-fold induction of reporter activity (Figure 5A). Ca^{2+} ionophore strongly synergized with Lck, leading to about 300-fold induction of NF-AT-LUC expression. On the other hand, no further induction was observed when Lck-transfected cells were costimulated with PMA. The induction of NF-AT-LUC by PMA+Ca^{2+} ionophore was maximal and was not further enhanced by Lck expression. We next examined whether the increase in NF-AT activity by Lck expression is mediated by a Ca^{2+}-dependent or -independent signal. About 50% inhibition of NF-AT-LUC induction by Lck was observed when the cells were pretreated with CsA (Figure 5B). These results suggest that activation of the NF-AT reporter by Lck and Syk relies only partly on a Ca^{2+}-dependent component, which most likely involves calcineurin.

Since Rac1 can potentiate JNK activation and induction of c-*jun* transcription by Syk, we examined its effect on induction of NF-AT-LUC expression by Syk. When Rac1(V12) alone was transfected with the NF-AT-LUC reporter, no induction of NF-AT activity was observed (Figure 5C). Rac1(V12) also did not potentiate the weak response to Lck. Rac1(V12) weakly potentiated (<2-fold) the strong response of NF-AT-LUC to coexpression of Syk.

A p38/JNK Inhibitor Partially Inhibits Syk- and Lck-Induced NF-AT Transcriptional Activity but Completely Inhibits c-*jun* Promoter Activation

To define whether JNK is necessary for the increase in NF-AT transcriptional activity induced by Lck and Syk,

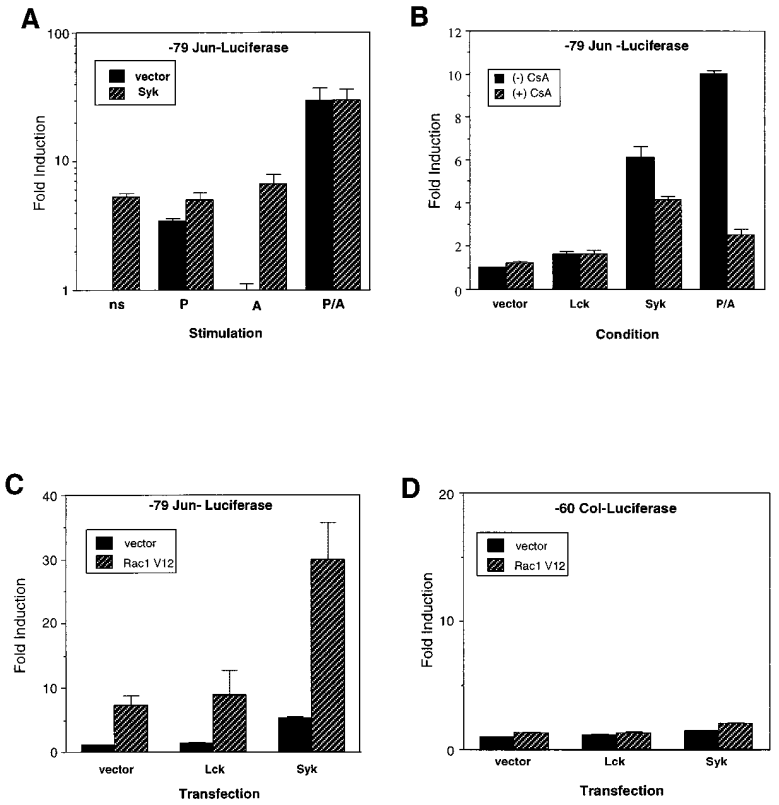

Figure 4. Syk Stimulates c-*jun* Promoter Activity

(A) Jurkat cells were cotransfected with 5 μg of either Syk or an empty expression vector along with 5 μg of −79 c-*jun*-LUC reporter plasmid. After 24 hr, cells were stimulated for another 12–16 hr as indicated and then harvested. Luciferase activity was measured and the results were expressed as average fold-induction relative to nonstimulated, vector-transfected cells (assigned an arbitrary value of 1). The averages of three experiments are shown using a logarithmic scale with standard error bars. ns, not stimulated; P, PMA; A, A23187; P/A, PMA+A23187.

(B) Jurkat cells were transfected with 5 μg of wild-type Syk, activated Lck, or empty vector together with 5 μg of −79 c-*jun*-LUC reporter. CsA (50 ng/ml) was added 24 hr after transfection. PMA and Ca^{2+} ionophore (P/A) were added as indicated 10 minutes after addition of CsA. Cells were harvested after incubation for another 12 hr and luciferase activity determined and expressed as in (A). Results shown are averages of three independent experiments.

(C) Jurkat cells were transfected with 5 μg −79 c-*jun*-LUC reporter and 5 μg of either Lck or Syk expression vector with or without 5 μg of a Rac1(V12) vector. Luciferase activity was measured as described in (A), and the results reflect averages of three separate experiments.

(D) Jurkat cells were transfected with 5 μg of −60 Col-LUC reporter and 5 μg of either Lck or Syk expression vector, with or without 5 μg of Rac1(V12) vector. Luciferase activity was measured as described in (A), and the results are averages of three separate experiments.

we used the compound SB202190, which was originally described as a specific p38 inhibitor (Lee et al., 1994). However, our laboratory has found that at levels that are 10-fold higher than those required for p38 inhibition, SB202190 is an effective JNK inhibitor (C.-Y. Chen and H. Le-Niculescu, unpublished data). Similar results were obtained with another p38 inhibitor SB203580 (Whitmarsh et al., 1997). Even when used at these higher concentrations, these drugs do not inhibit ERK activity. Incubation with 40 μM SB202190 diminished Lck- and Syk-induced NF-AT transcriptional activity by 50%, while induction by PMA+Ca^{2+} ionophore was almost completely inhibited by the drug (Figure 6A). Induction of NF-AT-LUC by cotransfection of activated PKC and calcineurin expression vectors was also inhibited by 40 μM SB202190 (data not shown). At 4 μM, which is sufficient for p38 but not JNK inhibition, SB202190 caused a considerably weaker inhibition of NF-AT-LUC induction. The effect of this inhibitor on NF-AT transcriptional activity is likely to be due to inhibition of AP-1 activity, because SB202190 completely inhibited induction of c-*jun*-LUC also at 40 μM (Figure 6B). As shown in Figure 6C, treatment of Jurkat cells with 40 μM SB202190 resulted in complete inhibition of c-Jun phosphorylation. Treatment with 4 μM SB202190 had only a marginal effect on c-Jun phosphorylation.

Discussion

MAPKs play an important role in the transduction of signals originating from cell surface receptors to the transcriptional machinery in the nucleus (Karin and Hunter, 1995; Marshall, 1995). In Jurkat T cells, occupancy of TCR/CD3 or incubation with PMA results in efficient ERK activation but has only a marginal effect on JNK activity. The latter requires costimulation provided by co-occupancy of CD3 and CD28 or simultaneous incubation with PMA+Ca^{2+} ionophore (Su et al., 1994). Such costimulation is also required for induction of IL-2 transcription (Linsley and Ledbetter, 1993). The correlation between costimulatory JNK activation and IL-2 induction suggests that activation of this group of MAPKs and possibly the related p38 MAPKs, whose regulation in T cells is very similar to that of the JNKs (E. J., unpublished data), is a critical component of the pathway leading to IL-2 induction. The significance of MAPKs in T cell activation is underscored by their diminished activation in anergic T cells, which correlates with inhibition of IL-2 synthesis (DeSilva et al., 1996; Li et al., 1996).

We investigated which signals are responsible for the differential regulation of JNK and ERK activities by focusing on events that occur downstream of PTKs that

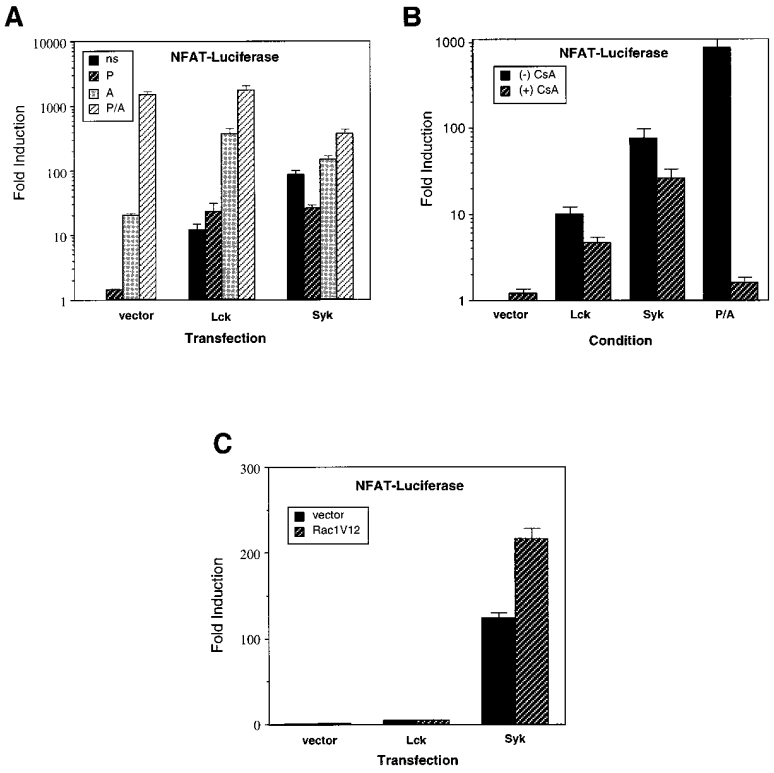

Figure 5. Lck and Syk Induce NF-AT Reporter Expression

(A) Jurkat cells were transfected with 2 μg of either Lck, Syk, or empty expression vector and 1 μg of an NF-AT-LUC reporter. Stimulations and determination of luciferase activity were performed as described for Figure 4. Average results from at least three independent experiments are shown using a logarithmic scale with standard error bars. ns, not stimulated; P, PMA; A, A23187; P/A, PMA+A23187.

(B) Transfections were performed as described above. After 24 hr, the cells were pretreated with CsA (50 ng/ml) for 10 min and then stimulated where indicated with PMA+A23187 (P/A) for 12–16 hr before harvesting. Luciferase activity was calculated and expressed as described in (A).

(C) Jurkat cells were transfected with NF-AT-LUC (1 μg) and 2 μg of either empty expression vector, Lck, Syk, or Rac1(V12) vectors. Luciferase activity was measured and expressed as described in (A).

participate in TCR signaling. Our studies demonstrate that PTKs of the Src and Syk families can stimulate both JNK and ERK but that cooperation with other signaling components, such as the small GTP binding protein Rac, results in enhanced JNK activation without an effect on ERK activity. The costimulatory effect of Rac is much more pronounced when combined with Syk rather than with Lck. The same results are obtained either when JNK activity is measured directly or through measurement of c-*jun* promoter activation, which depends on JNK activation (Karin, 1995). The lack of enhancement of ERK activity by Rac is consistent with the insensitivity of this MAPK to costimulation.

TCR stimulation results in recruitment and activation

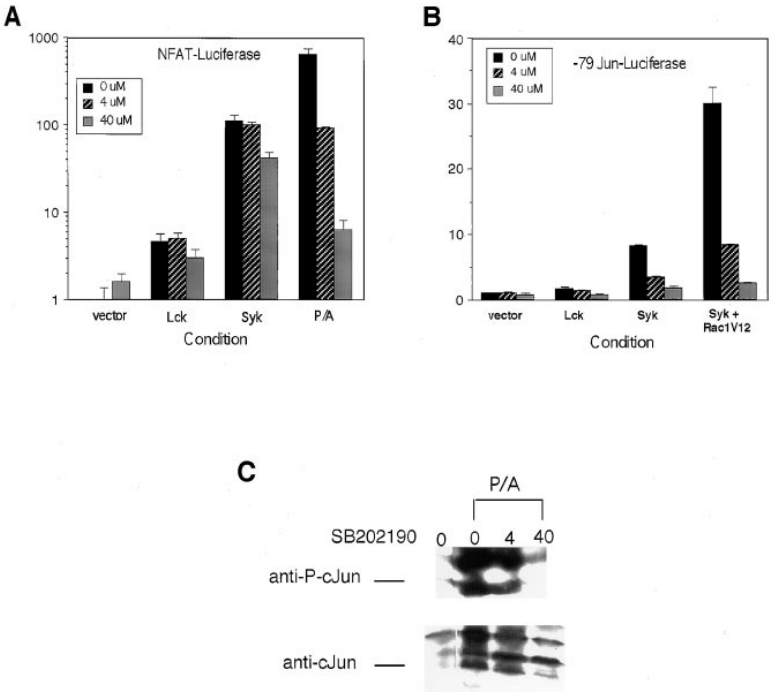

Figure 6. JNK Is Involved in the Syk and Lck Induction of NF-AT-LUC

(A) Jurkat cells were transfected with 2 μg of empty vector, Lck, or Syk expression vector together with 1 μg of NF-AT-LUC reporter. After 24 hr, cells were treated with vehicle alone or with either 4 or 40 μM of SB202190 added 15 min prior to PMA+Ca^{2+} ionophore (P/A) stimulation. Cells were harvested after 12 hr of incubation and assayed for luciferase activity. The averages of three experiments are displayed on a logarithmic scale with standard error bars.

(B) Jurkat cells were transfected with 5 μg of empty vector, Lck, or Syk expression vectors together with 5 μg of −79 c-*jun*-LUC reporter. The cells were treated with SB202190 and assayed for luciferase activity. Results are averages of three experiments.

(C) Jurkat cells were treated with 0.4 or 40 μM of SB202190. PMA+ Ca^{2+} ionophore (P/A) was added as indicated, 15 min later. Cells were harvested after 12 hr of incubation. N-terminal phosphorylation of c-Jun was examined by immunoblotting using anti-phosphoSer-73-c-Jun antibody. Total c-Jun expression was determined by probing the same blot with anti-c-Jun.

of PTKs. Phosphorylation of the ITAMs in the TCR is attributed to Src kinases, such as Lck and Fyn, which then trigger downstream signaling events including activation of MAPK cascades (Cantrell, 1996). The requirement for Lck in JNK activation through TCR/CD3 is clearly shown by the lack of response in the Lck-defective cell line (JCaM1.6). While Lck is necessary, it is not sufficient for full JNK activation, as observed in Lck-overexpressing cells (Figure 1A). Other studies have also suggested a requirement for Lck in CD28 signaling (Raab et al., 1995), but our results indicate that at least for JNK activation, Lck plays only a minor role in transmitting CD28-generated signals (Figure 2A).

Syk overexpression, on the other hand, enhances JNK activation by CD3, CD28, or PMA stimulation. Since all Jurkat (JE6)–derived clones, including the Lck-defective JCaM1.6, were found to express a truncated form of Syk (Fargnoli et al., 1995), the wild-type form of Syk is not absolutely required for JNK activation in response to TCR engagement in these cells. Although Syk and ZAP70 are differentially activated (Bu et al., 1995) and may have distinct roles (Kolanus et al., 1993; Chu et al., 1996), ZAP70, which is expressed normally in these cells (Chan et al., 1992), may compensate for the absence of functional Syk. Normal T cell signaling is also observed in mature T cells which express low levels of Syk and in Syk-deficient mice (Turner et al., 1995). However, in mice that are deficient in both ZAP70 and Syk, double-negative thymocytes fail to expand and differentiate into double-positive thymocytes (Cheng et al., 1997). Since this phenotype is not observed in mice deficient in only ZAP70 or Syk, these PTKs must have overlapping functions. Although transfection of wild-type ZAP70, unlike Syk, led to only weak JNK activation (Figure 1A), these results are consistent with the much lower level of ZAP70 basal kinase activity (Latour et al., 1996) and the presence of endogenous ZAP70 in these cells.

We examined whether GTPases that mediate PTK signals diverge in their regulatory function by differentially activating MAPKs in T cells. We found that although Ras activates ERK, it does not activate JNK even in the presence of other stimuli (data not shown). We also tested whether Ras can cooperate with Lck or Syk and found that it does not lead to enhanced activation of either JNK or ERK. This is in contrast to the strong effect of Ras on NF-AT transcriptional activity in the presence of Ca^{2+} ionophore (Genot et al., 1996; E. J., unpublished data). Different results were obtained by Faris et al. (1996), who found that inducible Ras(V12) in stably transfected Jurkat cells led to modest JNK activation. Currently, the basis for this discrepancy is not clear. Instead, as demonstrated in other cell types, Rac is more closely linked to JNK activation than Ras (Coso et al., 1995; Minden et al., 1995). In other cell types, Rac is regulated by signals from PTK receptors or the v-Src PTK (Coso et al., 1995; Minden et al., 1995). However, we did not observe a dramatic inhibition by dominant negative Rac1(N17) of Lck- and Syk-mediated JNK activation. It remains to be determined whether Rac mediates the response to another PTK in these cells. Our results suggest that in Jurkat cells, Rac can function in parallel to Syk, because it cooperates with it in JNK activation (Figure 3B). Since Syk-induced JNK activation can also be potentiated by CD3, CD28, or PMA stimulation (Figure 2B), it is possible that all of these stimuli act through Rac. Indeed, Rac1(N17) inhibited JNK activation by anti-CD3+anti-CD28, PMA+Ca^{2+} ionophore, or PMA+anti-CD28 (Figures 3D and 3E). Whether Lck, Fyn, or ZAP70 cooperate with other signals that lead to enhanced JNK activation remains to be examined. Of note, JNK activation by Rac1 is slightly attenuated by coexpression of Lck, suggesting the involvement of a Lck-dependent negative signal that impinges on the Rac pathway. Consistent with this, we observed decreased JNK activation by either anti-CD3+anti-CD28 or PMA+Ca^{2+} ionophore in Lck-transfected cells (data not shown).

We examined the effects of the different signaling proteins on AP-1– and NF-AT–dependent reporter gene expression. Although by itself Syk is a weak JNK activator, it is an efficient activator of the AP-1– and NF-AT–dependent reporters. The other PTKs—Fyn, Lck, and ZAP70—had only a weak effect on reporter gene expression (Figures 4 and 5 and data not shown). Our results point to the involvement of calcineurin in the Syk- as well as Lck-triggered pathways. Indeed, CsA partially blocked the Syk-induced activation of the NF-AT– and AP-1–dependent reporters. The CsA:cyclophilin complex targets and inhibits the activity of calcineurin, a Ca^{2+}-dependent phosphatase (Schreiber, 1992). Inhibition of calcineurin activity prevents nuclear translocation of NF-AT_c (Rao, 1994) and decreases Ca^{2+} dependent JNK activation and AP-1 transcriptional activity (Su et al., 1994; Werlen et al., submitted). Other studies have shown that clustering of a Syk chimera is sufficient to mobilize intracellular Ca^{2+} in both T and B cells (Kolanus et al., 1993; Takata et al., 1994). In JCaM cells that are defective in Ca^{2+} mobilization and express nonfunctional forms of Lck and Syk, reconstitution of either Lck or Syk expression can rescue Ca^{2+} signaling (Straus and Weiss, 1992; Chu et al., 1996), consistent with their overlapping functions in T cell development (Cheng et al., 1997). In addition to their role in Ca^{2+} signaling, Lck and Syk activate another component that leads to MAPK activation. This can be deduced from the partial inhibition of JNK activation by CsA and from the findings that JNK is not activated solely by calcineurin (or Ca^{2+} ionophore) and that calcineurin does not synergize with Rac to enhance JNK activation (Werlen et al., submitted). In addition, both Syk and Lck can activate ERK, independently of a Ca^{2+}-triggerred signal (Williams et al., 1997) (Figure 1B). Most likely, this second component involved in both JNK and ERK activation by Syk or Lck is PKC. Proteins such as PLC-γ, which is activated by PTKs (Weiss and Littman, 1994; Law et al., 1996) may mediate both their effects on PKC and Ca^{2+} mobilization.

The strong effect of Syk on NF-AT–dependent transcription is consistent with generation of both PKC- and Ca^{2+}-dependent signals, which induce NF-AT transcriptional activity. However, if Rac is transmitting these signals, it is paradoxical that Rac1(V12) does not induce NF-AT–dependent transcription by itself. This could be explained by our finding that Rac is specifically involved in JNK activation, such that a Ras-dependent signal (possibly mediated by ERK), which is crucial for NF-AT transcriptional activity (Cantrell, 1996), is not generated.

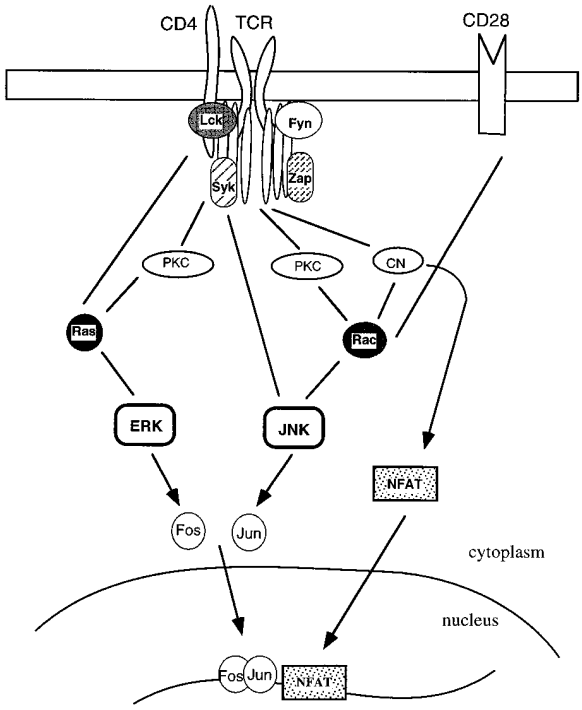

Figure 7. Signaling Pathways Leading to Stimulation of AP-1 and NF-AT Activities in T Cells

Our simplified model depicts multiple pathways generated by activation of PTKs leading to stimulation of AP-1 and NF-AT activities. A Ras-dependent pathway leads to ERK activation, while a Rac-dependent pathway leads to JNK activation. ERK and JNK are required for maximal induction of AP-1 activity, which cooperates with NF-AT$_c$ to bind the NF-AT element and activate NF-AT-dependent transcription. Syk generates signals that result in activation of PKC and calcineurin (CN), which converge on Rac, leading to JNK activation. Rac and Syk synergistically activate JNK, suggesting that Rac may account for coupling another pathway parallel to the one originating from Syk (such as from CD28) to JNK activation. Alternatively or at the same time, Syk may generate another signal (different from the one[s] leading to PKC and calcineurin activation) that synergizes with Rac, leading to potent JNK activation. Activation of CN also leads to dephosphorylation and translocation of NF-AT$_c$ to the nucleus, where it binds cooperatively with AP-1 to NF-AT response elements. In the case of AP-1 the signal is carried to the nucleus by ERK and JNK, leading to c-*fos* and c-*jun* induction.

An alternative explanation is that Rac is more intimately involved in the CD28 pathway, since CD28 by itself does not induce NF-AT or AP-1 transcriptional activities but can potentiate their activation by PMA+Ca^{2+} ionophore (Su et al., 1994; data not shown). The effect of Rac, as well as the effect of PMA and part of the effect of Syk on the NF-AT driven reporter, is most likely due to stimulation of AP-1 activity, which facilitates DNA binding of the Ca^{2+}- and calcineurin-responsive NF-AT$_c$ transcription factors (Rao, 1994). While the Ras-dependent signal that contributes to stimulation of NF-AT activity is probably mediated through ERK activation, leading to c-*fos* induction, the Ras-independent but Rac-dependent signal is likely to be propagated via JNK (and p38), leading to c-*jun* induction (Karin, 1995). In support of this interpretation, which is illustrated in Figure 7, we find that induction of the NF-AT-LUC reporter is partially inhibited by the nonsteroidal antiinflammatory drug SB202190 at a concentration that fully inhibits JNK activity. Since this drug has not been found to affect ERK activity, the Ras-mediated signal may account for the incomplete inhibition of the NF-AT-LUC activity. Indeed, SB202190 had a more potent effect on c-*jun* transcription, which is responsive to both JNK (Karin, 1995) and p38 (Han et al., 1997) activation.

Our results demonstrate that while both Lck and Syk can activate JNK and ERK, these pathways bifurcate, such that the Syk-generated signal converges with another signal that is Rac dependent, leading to enhanced JNK activation. In anergic T cells, a common regulatory signal that leads to both JNK and ERK activation in response to TCR/CD3 occupancy and that acts upstream of Ras is believed to be defective (Fields et al., 1996; Li et al., 1996). Our findings indicate that the regulation of JNK activity in T cells is dependent on Rac rather than Ras. Thus, a common regulator or activator of both small GTPases may be functionally impaired in anergic cells. A decrease in tyrosine phosphorylation has been demonstrated in restimulated anergic cells (Quill et al., 1992). In contrast, other TCR/CD3-induced events, such as NF-AT$_c$ dephoshorylation (Mondino et al., 1996), were generally unaffected in these cells, suggesting that a selective block in the MAPK–to–AP-1 pathway accounts for decreased IL-2 synthesis. Indeed, anergic cells display defective AP-1 activation (Kang et al., 1992). The cytoplasmic PTKs, particularly Lck, which is required for both JNK and ERK activation, are very likely targets for the mechanism responsible for T cell anergy. Definition of the signaling components leading from PTKs to MAPKs provides important clues regarding the mechanisms used by T cells to integrate and interpret the multiple signals that lead to activation or anergy.

Experimental Procedures

Antisera and Proteins

Anti-HA and anti-M2 antibodies have been described previously (Minden et al., 1995). Anti-JNK1 (333.8) and anti-Myc were obtained from PharMingen and anti-phospho-ERK from New England Biolabs. Anti-ERK2, anti-c-Jun, and anti-phospho-c-Jun were obtained from Santa Cruz Biotechnology. Anti-CD3 monoclonal antibody OKT3 was provided by Steve Hedrick, and anti-CD28 monoclonal antibody 9.3 was generously provided by Carl June and Bristol Myers Squibb. Lck polyclonal antisera and Ras monoclonal antibody were provided by B. Sefton. Expression and purification of glutathione S-transferase (GST)–c-Jun(1–79) have been described (Hibi et al., 1993). Myelin basic protein (MBP) was purchased from Sigma.

Expression Vectors, Reporters, and Transfections

The PTK expression vectors were tagged with the HA epitope by creating an NcoI site at the AUG codons of the different open reading frames by polymerase chain reaction mutagenesis. The mutated cDNAs were then subcloned into the SRα vector containing 3$\times$ HA epitope sequence (Minden et al., 1994). Lck expression vectors (wild-type Lck, Lck(F505), and HSB2-A1(Lck)) were gifts from M. Kamps and B. Sefton. Fyn expression construct (wild-type FynT) was provided by R. Perlmutter (Cooke and Perlmutter, 1989). The Fyn(F528) mutant was created by site-directed mutagenesis using the primer 5′-GCCACAGAGCCCCAGTTTCAGCCCGGTGAAAACCT GTG-3′ (Chameleon site-directed mutagenesis kit, Stratagene). Syk and ZAP70 expression vectors were gifts from A. Altman and A. Weiss, respectively. M2- and HA-tagged JNK and ERK constructs and Rac1(V12) and Rac1(N17) vectors have been described (Minden

et al., 1995). All constructs were subcloned into the pSRα vector (Minden et al., 1995). −79 c-*jun*-Luc and −60 Col-LUC reporters have been described (Deng and Karin, 1993; Su et al., 1994). NF-AT-LUC was provided by G. Crabtree (Northrop et al., 1993).

Tag-Jurkat cells, stably expressing SV40 large T antigen (a gift from G. Crabtree) were grown to about 10^6 cells/ml and concentrated to 2×10^7 cells/ml. Of this cell suspension, 500 μl was used for each transfection point. Cells were pulsed using BioRad GenePulser at 250 V, 960 μF in a 0.4 cm cuvette. Cells were incubated on ice for 10 min and then resuspended in 10 ml of complete RPMI medium. For reporter gene assays, cells were stimulated 24 hr after transfection and then harvested after another 12 hr of incubation. Cells were lysed in 100 μl of buffer II (100 mM Tris-Ac [pH 7.8], 10 mM MgAc, 1 mM EDTA) with 1% Triton X-100 and 1 mM dithiothreitol. Luciferase activity was determined using luminometer (MicroLumat, EG&G Berthold). For kinase assays, cells were harvested 36 hr after transfection.

Cell Culture and Stimulations

Jurkat wild-type and variant cells (JCaM1.6 and Tag) (gifts from A. Weiss and G. Crabtree, respectively) were grown in RPMI with 10% fetal calf serum, 1 mM glutamate, 100 U/ml penicillin, and 100 mg/ml streptomycin (complete medium) at 37°C with 5% CO_2. When indicated, cells were pretreated with 100 ng/ml CsA (Sandoz) or SB202190 (SmithKline Beecham) for 10–15 min. Cells were then stimulated with 10 ng/ml PMA from Sigma, 0.5 μg/ml A23187 (Calbiochem), or 10 μg/ml anti-CD3, 2 μg/ml anti-CD28 (in solution) either alone or in combination for 30 min before harvesting. Cells were lysed in 200 μl of whole-cell extract (WCE) lysis buffer (Hibi et al., 1993).

Immunoprecipitation and Immunoblotting

For immunoblotting, 30 μg of cell lysate were resolved by 10% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and transferred onto Immobilon-P membrane (Millipore). After blocking with 5% milk in phosphate-buffered saline (PBS), the membrane was washed with PBS-T (0.2% Tween-20) and then incubated with the appropriate primary antibody (1:2000 dilution) for at least 1 hr at room temperature. The membrane was washed again with PBS-T once for 15 min and twice for 5 min before being incubated with secondary antibody conjugated to horseradish peroxidase. The membrane was thoroughly washed with PBS-T and visualized with an enhanced chemiluminescence detection system (Amersham). The amount of expressed protein was quantitated by phosphoimager (Bio-Rad) and the quantity of lysate used for kinase assays was adjusted if necessary.

For immunoprecipitations, cell lysates (50 μg of protein) were immunoprecipitated with the appropriate antibody (0.5 μl of anti-HA or anti-M2) using protein A or G as needed in a total of 200 μl of WCE lysis buffer and rotated for 2 hr at 4°C.

Protein Kinase Assays

Immunoprecipitated proteins bound to Protein A or G beads were washed twice with WCE lysis buffer and then once with kinase buffer before incubation with 5 μg of GST-c-Jun(1–79) or 2 μg of MBP and [γ-^{32}P]ATP (2–5 μCi) in kinase buffer (Hibi et al., 1993). Proteins were resolved on 10% or 13% SDS-polyacrylamide gels for JNK and ERK assays, respectively. The fold activation of JNK or ERK was quantitated using a phosphoimager (Bio-Rad).

Acknowledgments

We thank A. Altman, G. Crabtree, S. Hedrick, C. June, M. Kamps, J. Lee, A. Minden, R. Perlmutter, B. Sefton, and A. Weiss for providing the necessary constructs and reagents. We are also grateful to Bristol Myers Squibb, SmithKline Beecham, and Sandoz for providing reagents as mentioned. We thank Martina Pasillas for technical help with the oligo synthesis and Todd Giles for manuscript preparation. E. J. was supported by a minority supplement to National Institutes of Health (NIH) grant CA50528. G. W. was funded by the Swiss Academy of Medical Sciences and Swiss National Science Foundation grant 823A-046706. This work was supported by NIH grants CA50528 and ES04151.

Received September 18, 1997; revised November 17, 1997.

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